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The Dunham-Bujwid indol test, used originally to distinguish cholera spirilla from other spirilla, was applied by Kitasato to bacteria other than those of Asiatic cholera, notably the colon bacillus and the typhoid bacillus, and to-day the test for indol is, by common consent, used regularly in the study of bacteria. As a result of its application we now know of two classes of indol-producing bacteria—those which produce, in addition, nitrites, and those which do not. The first includes the spirillum of Asiatic cholera; the second, most other indol-producing species. The fairly extensive literature deals mainly with the first class, because of the obviously important bearing of any method which will distinguish cholera from other spirilla. As is well known, the "cholera-red" reaction is brought out by adding sulphuric or chlorhydric acid free from nitrous acid to bouillon cultures, when the presence of indol plus nitrites will impart a violet-red color to the fluid. Dunham introduced the nutritive solution consisting of 1 per cent peptone and ½ per cent sodium chloride in water. This is now universally used in testing for indol.

There have been not a few voices raised in criticism of this method for determining the cholera-red reaction. To these I shall refer only as they antedate my own efforts towards improving the indol test, since I have restricted myself to the purely indol-producing forms. In attempting for a number of years past to apply the indol test by using the simple peptone solution I have been forced to consider the method quite unsatisfactory for some groups of bacteria. I was therefore led recently to try dextrose-free bouillon, with a premonition that possibly the carbohydrate may inhibit the production of indol as it does that of toxins in some cases. The result was very satisfac-

tory. Other facts were brought out at the same time which tend to explain the failure of the test under certain conditions. A search through the literature showed me that the restraining influence of sugar had been already demonstrated. Gorini,* in discussing the uncertainty of the cholera-red reaction, states that in the presence of 0.5 per cent dextrose, saccharose and lactose, cholera spirilla fail to give the red reaction. He lays the blame upon the presence of traces of sugar in the peptone and gives a number of tests by which a pure peptone may be recognized. Kruse† briefly refers to this subject by stating that when bouillon contains more than 0.25 per cent sugar the indol reaction no longer appears. He adds that cultures should remain at least a week in the thermostat, a point brought out in the experiments to be given below. These are the only references I can find which bear directly upon my own observations. These I shall very briefly summarize.

1. The peptone‡ solution is a very poor culture fluid for many bacteria; some fail to grow in it. Dextrose-free bouillon is far better. All bacteria tried grew in it, some quite vigorously. In the dextrose-free bouillon the indol reaction is pronounced in 16-hour cultures, while in peptone solution it required from three to six days for traces of indol to form. The coloration of the dextrose-free bouillon becomes intense, usually within a few seconds after the nitrite and acid are added. In the peptone solution it remains feeble, often so much so that a decision is impossible.

2. In bouillon containing muscle sugar the indol reaction becomes positive after the bacteria have converted the sugar into acid and nearly neutralized the latter by alkali production. If the bacteria are unable to neutralize the acid formed, either because too much sugar was present or because they are inherently unable to produce much alkali, the indol reaction will fail. This is well illustrated by two indol-producing groups, the colon and the rabbit-septicæmia group, the former a rapid, abundant alkali producer, the latter not.

* Anmerkung ü. d. Cholerarotreaktion. *Centralbl. f. Bakt.*, xiii (1893), 790.

† Kritische u. exp. Beiträge z. hygienischen Beurtheilung d. Wassers. *Zeitschr. f. Hygiene*, xvii (1894), 1.

‡ That used was from two lots of Witte's peptone.

Comparative experiments were made by inoculating a series of tubes, one a day, for eight or nine days. All were tested for indol at the same time, so that the time element in the formation of indol might not be neglected. Each tube (cotton-plugged) received about 1 cc. of a 0.01 per cent solution of KNO_2 freshly prepared and 10 drops of chemically pure H_2SO_4.

With B. coli in a bouillon containing enough muscle sugar to greatly inhibit the multiplication of diphtheria bacilli, the indol reaction appeared intensely after the acid formed had been sufficiently neutralized by alkali production to give a feebly blue color with neutral litmus, or when the acidity, according to phenolphthalein, was about 1 per cent of a normal solution. This point was reached on the third or fourth day. Younger cultures gave only a feeble tint. On the other hand a culture of fowl cholera bacteria (rabbit-septicæmia group) in bouillon containing muscle sugar failed to give any indol reaction in any tube of the series. The reaction remained acid to litmus. The same culture gave an intense violet-red color in dextrose-free bouillon after 18 hours in one series, 48 hours in another.

When Dunham's solution was used the fowl cholera bacteria gave a feeble but easily recognizable tint in the tube 9 days old, but none in the 7 younger cultures. The colon bacillus gave a well-marked violet-red color in tubes older than 3 days, no distinct change in those younger than 3 days. In dextrose-free bouillon a deep violet-red was brought out in 18-hour tubes, growing more intense in cultures according to their age. Tubes less than 18 hours old have not been tried.

Cultures of B. coli in the fermentation tube were also tested. In former publications I have pointed out that while the entire fluid becomes acid when sugar is present, the fluid in the closed branch remains so, and that in the open bulb in free contact with oxygen becomes alkaline provided the sugar does not exceed a certain amount (0.3 to 0.4 per cent, approximately, for B. coli). In such cultures the acid fluid in the closed branch gave a negative, the alkaline fluid in the bulb a positive indol reaction.*

* Since the acid reaction remains while the anaerobic conditions persist, it would seem as if indol were not producible by facultative-anaerobic bacteria, such as B. coli, except in contact with oxygen, unless the method of demonstrating indol, here used, is at fault.

3. From what precedes it is evident that mere traces of dextrose in peptone cannot be held responsible for the feeble indol reaction, as claimed by Gorini. A two per cent peptone solution tested in the fermentation tube showed only the faintest growth in the closed branch when inoculated with B. coli. It is furthermore evident that sugars inhibit indol production only under certain conditions, varying with the organism studied. The advantages of the dextrose-free bouillon over the peptone solution may be due either to its greater general nutritive value or to the presence of substances yielding indol more readily than does the peptone.

The preparation of dextrose-free bouillon is simple enough. Some years ago the writer selected such bouillon from that made in the ordinary way by testing every lot in the fermentation tube. This method is unsatisfactory, because most beef contains more than traces. Spronck's method, of allowing beef to decompose for several days, is unreliable because the bouillon made from it frequently contains relatively much sugar. The procedure now used by me is uniformly successful. Beef infusion, prepared either by extracting in the cold or at 60° C., is inoculated in the evening with a rich fluid culture of some acid-producing bacterium (I use temporarily B. coli) and placed in the thermostat. Early next morning the infusion, covered with a thin layer of froth, is boiled, filtered, peptone and salt added and the neutralization and sterilization carried on as usual. Such bouillon when tested with B. coli in the fermentation tube will no longer permit any anaerobic growth. The closed branch remains clear, a sign that carbohydrates are absent.* It might be claimed that such bouillon would normally contain indol. This is not the case. Its absence was shown by the negative indol reaction of the sterile bouillon and by the negative indol reaction of bacteria, such as typhoid, hog cholera (several varieties), mouse septicæmia, B. lactis aerogenes and diphtheria,† of which series were tested as detailed above.

* T. Smith. Ueber d. Bedeutung des Zuckers in Kulturmedien f. Bakterien. *Centralbl. f. Bakt.*, xviii (1895), 1.

† Contrary to Palmirski and Orlowski (*Centralbl. f. Bakt.*, xvii (1895), 358), who claim that the diptheria bacillus produces indol.

Nitrites were likewise absent, for the indol-producing bacteria did not give the indol reaction when H_2SO_4 alone was added.

Questions bearing on the production of indol in the presence of different kinds of peptones, and of sugars not acted upon by the bacteria under examination, as well as in bouillon free from peptone, are left untouched. The effect of the change of procedure upon the cholera-red reaction is likewise abandoned for the present, because cholera cultures are not available.